UPTAKE OF RADIOACTIVE GLUCOSE AND AMINO ACIDS AND THEIR UTILIZATION FOR INCORPORATION INTO PROTEINS DURING MATURATION AND FERTILIZATION OF THE EGGS OF ASTERIAS FORBESII AND SPISULA SOLIDISSIMA[1]

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One of the factors considered to be responsible for the different respiratory response to fertilization of the eggs of different animals is the stage of maturation at which fertilization takes place (for a discussion, see Rothschild, 1956; Monroy, in press). This evidently implies also a different metabolic condition of various eggs at the moment of fertilization. On the contrary, according to one of the present authors (Monroy, in press), all eggs are fertilized in a fundamentally similar physiological condition, independent of the stage of maturation attained. It has been suggested that in the course of the intra-ovarian maturation a metabolic block builds up progressively within the egg. When the threshold concentration of the inhibiting factor(s) is reached, maturation is arrested. The stage to which nuclear maturation has progressed would thus indicate the moment when such a threshold concentration has been attained.

Hence, at fertilization all eggs should be released from a metabolic inhibition. If this is correct, and the results to be presented here suggest that it may indeed be so, the next question is: does the release of the inhibition immdiately follow fertilization or does it require that maturation be completed first in the case of those eggs which are fertilized before the initiation or the completion of maturation? The present experiments provide also some information on this question.

One of the most impressive indications of the metabolic inhibition of the unfertilized sea urchin egg is its inability to carry out protein synthesis. In fact, although unfertilized sea urchin eggs take up labelled amino acids, these are not (or to a negligible extent) incorporated into proteins (Hultin, 1950, 1952; Hoberman, Metz and Graff, 1952; Nakano and Monroy, 1958; Monroy, 1960). Particularly striking have been the results obtained with C^{14}-glucose, which is taken up by the unfertilized egg and used for the synthesis of alanine, serine, aspartic and glutamic acid. However, no incorporation of these amino acids into proteins occurs prior to fertilization (Monroy and Vittorelli, 1962). Incorporation into proteins of amino acids either administered from without or already present in the egg begins immediately following fertilization. On the other hand, incorporation of amino acids in the proteins is quite active in the oocytes (see Monroy and Maggio, in press), which is in agreement with the notion of the high rate of oxygen consumption of the oocyte and of its decline during maturation (Lindahl and Holter, 1941).

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In the experiments presented in this paper the rate of incorporation of labelled amino acids and of C^{14} from glucose into the proteins has been investigated in the eggs of *Asterias forbesii* and *Spisula solidissima*. The ripe eggs of *Asterias* are shed with an intact germinal vesicle which ruptures as soon as the eggs reach the sea water. Maturation then progresses until the formation of the two polar bodies. The eggs can be inseminated at any moment during this cycle although the percentages of fertilization and cleavage are higher when the eggs are fertilized between the rupture of the germinal vesicle and the formation of the first polar body. Fertilization is accompanied by the formation of the fertilization membrane but the sperm nucleus remains "idle" beneath the egg cortex until the completion of maturation.

In the eggs of *Spisula*, on the contrary, the breakdown of the germinal vesicle, *i.e.*, the beginning of maturation, is induced by fertilization.

The eggs of *Asterias* and *Spisula* were selected for experiment because of the different conditions under which maturation is initiated; this should permit useful comparison with the previous results obtained with the sea urchin egg. A preliminary report of these results has been already published (Tolis and Monroy, 1963a, 1963b).

Material and Methods

Preparation of the egg

Ripe gametes were obtained from *Asterias* by an injection of a preparation of the spawning factor of Chaet (1964); spawning followed within 30–45 minutes. The eggs were suspended in sea water, centrifuged gently, re-suspended in sea water and divided into two equal batches. One batch was left unfertilized while the other was fertilized; both batches were allowed to stand in large fingerbowls at about 18° C., and measured aliquots were collected at the desired times for the exposure to the radioactive compounds. The time elapsed between spawning and fertilization usually did not exceed 10 minutes. One hundred per cent fertilization was the rule; normal cleavage occurred in 80–95% of the eggs. Under the conditions of our experiments the formation of the polar bodies occurred simultaneously in the unfertilized and fertilized eggs, the first being formed about 40 minutes after shedding and the second about 20 minutes later. Because of the large quantities of eggs obtained, the eggs of a single female were used for each experiment. Eggs were artificially activated by a 5-minute treatment with the Ca-Na hypertonic sea water as suggested by R. S. Lillie (1941), *i.e.*, 10 ml. of 2.5 *M* Ca-Na solution (95 ml. of 2.5 *M* NaCl + 5 ml. of 2.5 *M* $CaCl_2$) + 50 ml. sea water.

The gametes of *Spisula* were obtained and handled as suggested by Allen (1953). Under these conditions, 100% fertilization and cleavage were obtained. The eggs were cultured in a thin layer in large fingerbowls. Breakdown of the germinal vesicle took place between 9 and 12 minutes after fertilization; the first polar body was formed about 30 minutes after fertilization and the second 20 minutes later.

Pulse labelling

The eggs were exposed to the radioactive compounds at certain stages during maturation and fertilization. In the experiments with labelled amino acids, strong labelling was obtained with 5-minute pulses, whereas in the case of glucose an exposure of 30 minutes was necessary. Hence in the latter case the definition of the stages was less precise. In the case of *Asterias* the first pulse was given as soon as in the batch of fertilized eggs the fertilization membrane appeared; then at the time of the formation of the first and second polar bodies and finally twice more during cleavage (see figures). In the experiments with *Spisula* eggs the germinal vesicle breakdown stage was also included.

The following procedure was followed throughout the experiments both with *Asterias* and *Spisula*. A measured aliquot of egg suspension was pipetted into a 10-ml. Erlenmeyer flask; the solution of the labelled compound was then added to a final concentration of: C^{14}-D-glucose (U):0.5 μC./ml. (specific activity 3.83 μC./ /μM); S^{35}-L-methionine:1 μC./ml. (specific activity 13.4 μC./μM); L-valine-1-C^{14}:1 μC./ml. (specific activity 5.9 μC./μM). The labelled compounds were added simultaneously to the flasks of the fertilized and unfertilized eggs. In the glucose experiments at the end of the treatment, the eggs were rapidly centrifuged (a hand centrifuge has been used for such washings), washed once with ice-cold sea water containing 1% C^{12}-D-glucose and three times with sea water. In the amino acid experiments, the uptake was stopped by the addition of an excess of cold amino acid in sea water to bring the final concentration to 1 mg./ml.; after centrifugation, the eggs received three more washings with sea water. Further processing was the same in all experiments. The whole washing procedure did not take more than 10 minutes. The packed eggs were taken up in 1 ml. of ice-cold distilled water and transferred to a homogenizer in which they were rapidly homogenized. From the homogenates, two 0.1-ml. samples were collected for the estimation of total nitrogen and three 0.1-ml. samples were rapidly dried on filter paper discs. One of the discs was used for the estimation of total uptake; the other two were extracted with TCA and alcohol-ether, as suggested by Mans and Novelli (1960). Counting was done in a Packard scintillation counter at 50% efficiency. Total nitrogen was estimated by Nesslerization after combustion.

RESULTS

1. *Asterias forbesii*

(a) *Experiments with C^{14} glucose.* Preliminary experiments carried out by the senior author on the egg of the Mediterranean *Asterias glacialis* showed that both unfertilized and fertilized eggs were able to utilize the carbon from glucose for the synthesis of glutamic and aspartic acid and alanine. The curves of Figure 1 show that in the unfertilized eggs the rate of total uptake underwent a sharp decline until after the formation of the second polar body and then remained almost constant. In the fertilized eggs, during the first half hour after fertilization (approximately until the formation of the first polar body) the rate of uptake was lower than in the unfertilized eggs, but then it did not undergo the rapid drop observed in the unfertilized eggs. Indeed, it remained almost constant until completion of maturation and then began slowly to increase. The significance of the lower rate of uptake of the fertilized eggs during the first half hour following fertilization is at present obscure. A small increase in the rate of incorporation into the proteins during maturation was sometimes observed both in the unfertilized and fertilized eggs (as in the experiment used for Figure 1). In other cases (as in the experiment used in Table I) no such increase occurred until after the formation of the second polar body. After the completion of maturation, the rate of incorporation rose in the fertilized eggs, whereas it remained either constant or declined in the unfertilized eggs. It must be noted, however, that the values of

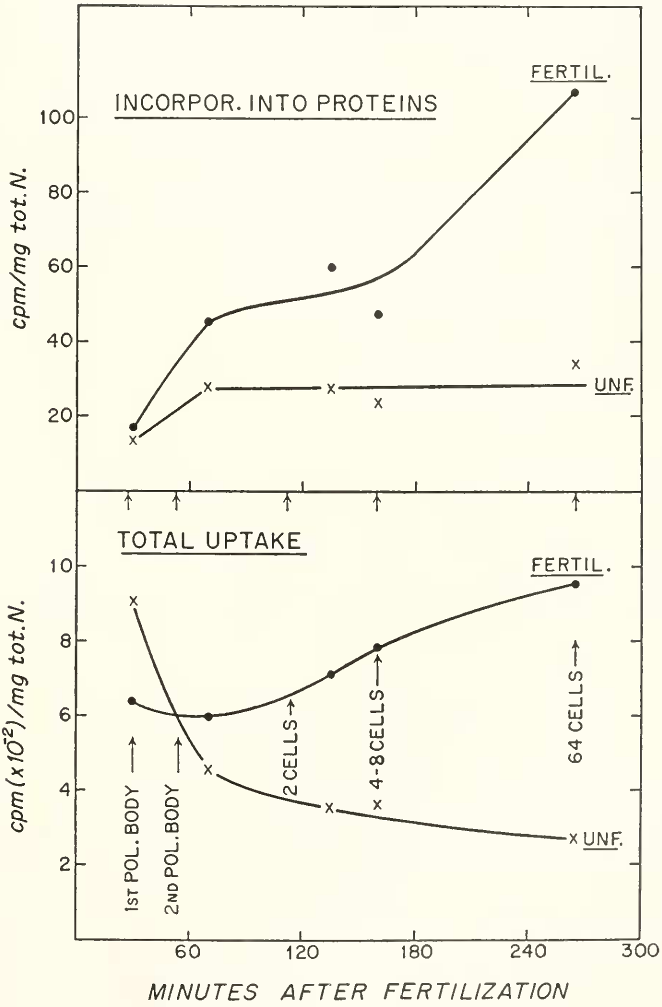

FIGURE 1. *Asterias forbesii.* Rate of total uptake and incorporation into proteins of C^{14} from glucose. The position of the points of the curves is at the end of the 30-minute pulse. For the interpretation of the incorporation curve see the text.

incorporation were always fairly low and therefore the only safe statement that can be made concerns the increase that occurs in the fertilized eggs after maturation has been completed. More extensive experiments are required to check the actual shape of the incorporation curve during cleavage.

Spontaneous activation of the unfertilized eggs was observed in some experiments. Activation was indicated by the elevation of a fertilization membrane and centralization and swelling of the nuclei. Activation was always accompanied by a sudden and transient increase in the rate of total uptake. The rate of incorporation into the proteins, on the contrary, did not change appreciably (Table I). Experiments of parthenogenetic activation were carried out using the hypertony method of R. S. Lillie (see Methods). Artificial activation, accomplished soon after the eggs had been shed (*i.e.*, following the same schedule as in the fertilization experiments), was followed by an immediate increase in the rate of uptake.

TABLE I

Rate of uptake and of incorporation into the proteins of C^{14} from glucose in spontaneously (column 1) and artificially (column 4) activated Asterias eggs. Spontaneous activation took place independently of any treatment, in the course of an experiment at the indicated time; artificial activation was carried out 10 minutes after shedding. In both experiments time is indicated from fertilization or artificial activation.

The data in column 1 should be compared with those in column 2 (fertilized eggs of the same batch), and those in column 4 with those in column 3 (unfertilized eggs of the same batch). Radioactivities are expressed as C.P.M./mg. total nitrogen.

Time	1 Unfertilized		2 Fertilized		Time	3 Unfertilized		4 Activated	
	Uptake	Incorp.	Uptake	Incorp.		Uptake	Incorp.	Uptake	Incorp.
30	1010	15.1	935	19.3	30	810	31.2	1565	42.6
60	650	16.4	814	15.5	150	585	9.8		
140	496	lost	930	40.5					
	Activ.—				210	481	3.3	1605	64.2
190	920	15.0	1214	73.5					
300	733	7.2	1260	72.5					

Thus, whereas in the fertilized eggs there is a transient period during which the permeability appears to be lowered, in the activated ones it always undergoes an immediate increase. This seems to suggest a different type of surface change in the activated with respect to the fertilized egg. On the contrary, the increase in the rate of incorporation into the proteins was smaller than in the fertilized eggs (Table I). Also in the sea urchin eggs the amino acid incorporation into proteins in butyric acid-activated eggs was lower than in the fertilized ones (Nakano, Giudice and Monroy, 1958).

(b) *Experiments with S^{35}-L-methionine and L-valine-1-C^{14}.* The experiments with valine (Fig. 2) duplicate quite well the results obtained with glucose. Indeed, in these experiments a decline of the rate of uptake was observed during maturation, which was less pronounced in the fertilized than in the unfertilized eggs. Again the first pulse (which, as mentioned, in these experiments was of 5

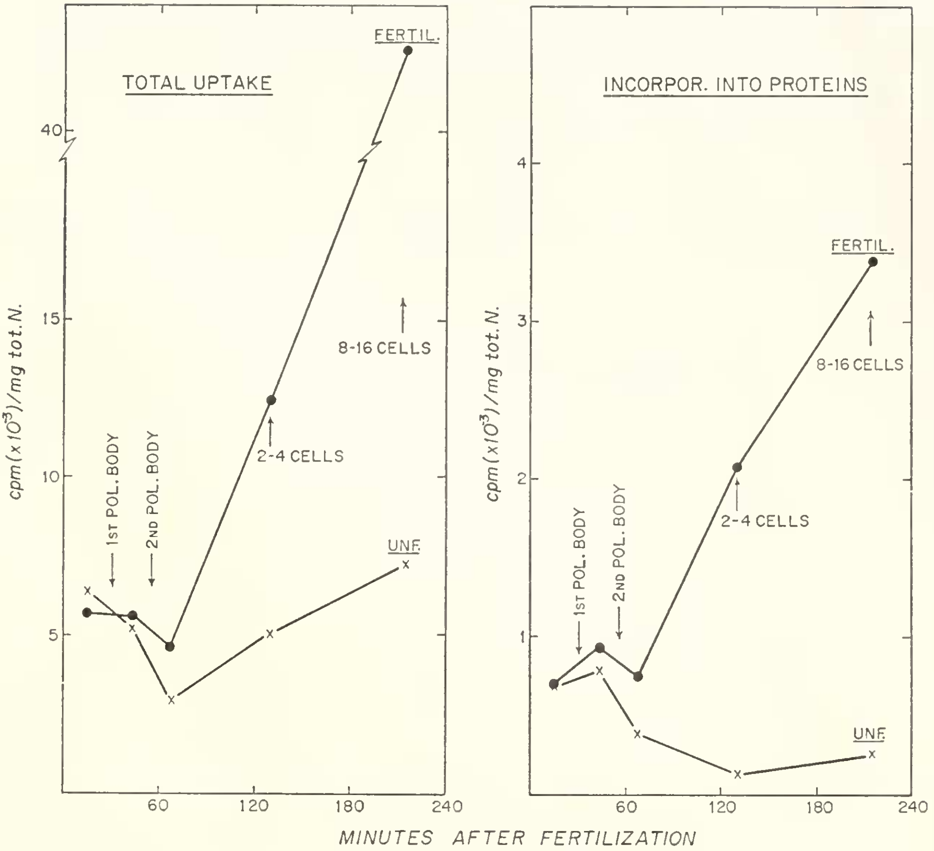

FIGURE 2. *Asterias forbesii.* Rate of total uptake and incorporation into proteins of L-valine-1-C^{14} in unfertilized and fertilized eggs. At the time of the fourth pulse, 40% of the unfertilized eggs had undergone activation and at the time of the last pulse nearly 90% had membranes and centralized and swollen nuclei.

minutes) showed a slightly lower rate of total uptake in the fertilized than in the unfertilized eggs. After completion of maturation the increase in the rate of total uptake in the fertilized eggs was far greater than in the glucose experiments. In the experiment used for the preparation of Figure 2, the unfertilized eggs underwent spontaneous activation soon after the end of maturation and also in this case a slight increase of the rate of uptake took place. In another experiment in which this did not take place, no such increase was observed.

When methionine was tested (Fig. 3) the rate of uptake in the unfertilized eggs increased slightly but linearly during the four hours of the experiment; no change was observed in conjunction with the completion of maturation. Furthermore, until the formation of the second polar body, the values of the rate of uptake were practically identical in the unfertilized and in the fertilized eggs. Afterwards a dramatic increase was observed in the fertilized egg. Both in the unfertilized and fertilized eggs the rate of incorporation of valine and methionine into proteins increased slightly during maturation. Once maturation was completed, the rate of incorporation declined in the unfertilized eggs while it increased considerably in the fertilized ones.

2. *Spisula*

Only experiments with valine were performed on these eggs. A typical experiment is summarized in Figure 4. In the unfertilized egg the rate of uptake re-

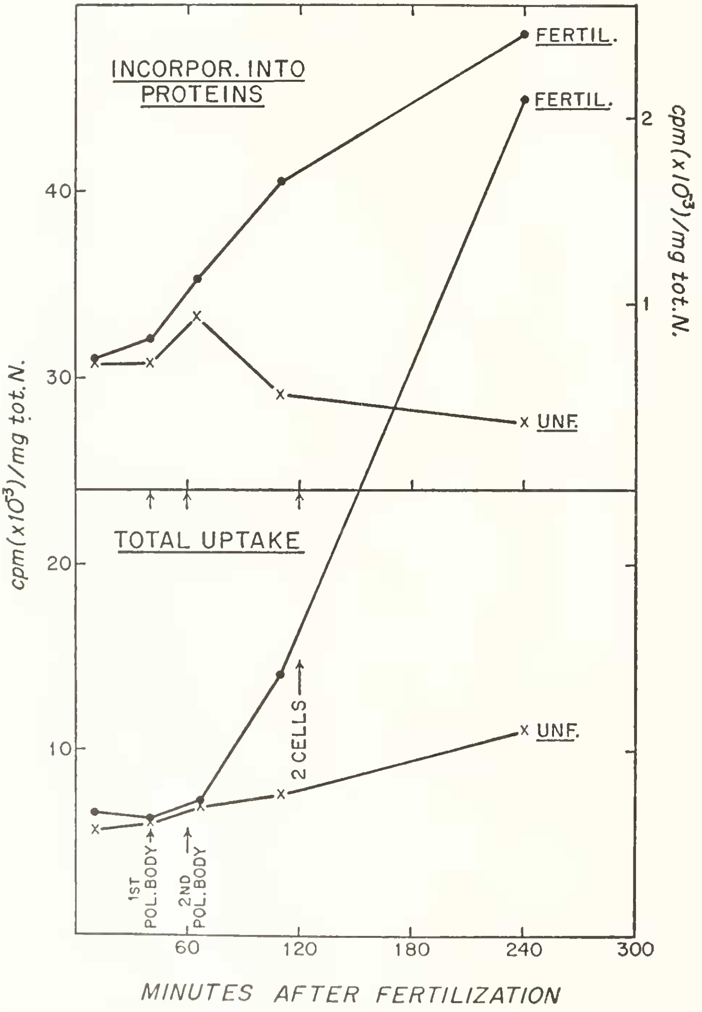

FIGURE 3. *Asterias forbesii.* Rate of total uptake and incorporation into proteins of S^{35}-L-methionine. The figures on the left ordinate refer to the total uptake and those on the right one to the incorporation into proteins.

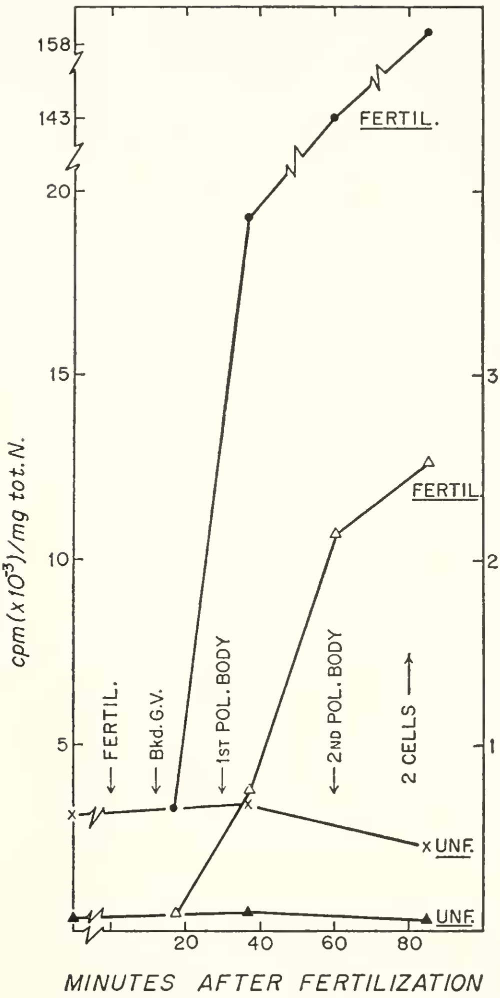

FIGURE 4. *Spisula solidissima.* Rate of total uptake (x——x, unfertilized eggs; ●——●, fertilized eggs) and incorporation into proteins (▲——▲, unfertilized eggs; △——△, fertilized eggs) in eggs pulsed with L-valine-1-C^{14}. Values on the left ordinate refer to total uptake and on the right ordinate to incorporation into proteins.

mained almost constant for about 60 minutes and then declined slightly. The rate of incorporation into proteins was low and remained constant (Fig. 4). When a 5-minute pulse was given to fertilized eggs in which the germinal vesicle had just broken down (the breakdown of the germinal vesicle usually started about 10 minutes after insemination and was complete in about 5 minutes) the rate of total uptake and of incorporation was the same as one would expect in the unfertilized eggs (Fig. 4). Indeed, the experimental points fall exactly on the expected line of the unfertilized eggs. However, a pulse applied about 20 minutes later (*i.e.*, 5 minutes after the formation of the first polar body) showed a great increase in the rate both of uptake and of incorporation (Fig. 4). Indeed at the completion of maturation the rate of uptake was more than 40 times greater than that in the unfertilized egg, and the rate of incorporation into proteins had increased 20 times. The increase continued during the first cleavage but was not as rapid as during maturation.

DISCUSSION

The results of the experiments described in this paper support the hypothesis that at the moment of fertilization all the eggs, irrespective of the stage of maturation reached, are in a metabolically inhibited condition. In fact both the eggs of *Asterias* and of *Spisula*, in spite of their being shed as primary oocytes, have proved to have very little, if any, ability to carry out incorporation of amino acids into proteins. Hence they are in an analogous condition to the fully mature sea urchin egg before fertilization. A comparison of these results with the respiratory metabolism may prove especially illuminating. Nothing is known about the respiratory changes of the *Spisula* egg following fertilization. As for the *Asterias* egg, according to Borei (1948), at the onset of maturation the rate of oxygen consumption begins to increase; it reaches a peak at the time of the first meiosis and then starts to decline. Hence in this respect the starfish egg would appear to behave differently from the sea urchin (Lindahl and Holter, 1941) and fish (Nakano, 1953) oocytes, in which the rate of oxygen consumption rapidly declines at the onset of maturation. Following fertilization the respiratory rate increases slowly. However, if the slower rate of cleavage of the starfish as compared to the sea urchin egg is taken into consideration, then the two respiratory curves turn out to be well superimposable (Borei and Lybing, 1949). In our experiments, a decline in the rate of uptake of glucose and valine during maturation has been observed both in the unfertilized and fertilized eggs whereas in the methionine experiments it remained practically constant. Thus, the curves for glucose and valine are similar to the respiratory curves of the maturing sea urchin and fish oocytes. On the other hand, the curve of incorporation into the proteins is similar to the respiratory curve described by Borei (1948) in starfish. It must be emphasized that work with starfish oocytes and eggs has been hampered by the difficulty of obtaining homogeneous batches of eggs. Now that this difficulty has been largely overcome (Chaet, 1964), a re-examination of the respiration of the starfish oocyte during maturation and fertilization becomes imperative. However, another possibility to explain the decrease in the uptake of glucose and valine during maturation is that it may simply depend on the decreased permeability of the egg upon standing in sea water; such a decrease would apparently be somewhat lessened by fertilization. This possibility is born out of the observation of Dalcq (1924) that the resistance of the starfish oocyte to cytolysis by dilute sea water increases during maturation. The acceleration of incorporation observed during maturation both in the unfertilized and in the fertilized eggs is worth mentioning. Whether or not this acceleration is connected with the maturation divisions (formation of the spindle?) is at present obscure.

The present experiments have also provided some information pertaining to the second question raised in the introduction, namely whether the inhibition of the unfertilized egg is released immediately following fertilization, as in the case of the sea urchin egg, or if in the eggs which are fertilized before the initiation or in the course of maturation, this must be completed first. In this respect, the eggs of *Asterias* and of *Spisula* have behaved differently. In fact, in the former the release occurs only after the completion of maturation, in the latter soon after the breakdown of the germinal vesicle and well before the formation of the first polar body. Our data, although limited to the eggs of only two animal species, show that the inhibition is not released *immediately* following fertilization. Indeed, maturation must progress to a certain stage, different in the eggs of the different animal groups, for such a release to occur.

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SUMMARY

1. In the eggs of *Asterias forbesii* the rate of total uptake of C^{14}-glucose and of L-valine-1-C^{14} declines during maturation both in unfertilized and fertilized eggs. The rate of uptake of S^{35}-L-methionine remains constant throughout maturation. After completion of maturation the rate of uptake is greatly increased in the fertilized eggs, whereas in the unfertilized ones it either continues to decline or remains constant.

2. The rate of incorporation of C^{14} from glucose and of C^{14}-valine and S^{35}-methionine into proteins undergoes a slight acceleration during maturation both in unfertilized and fertilized eggs. After the formation of the second polar body, the rate of incorporation declines in the unfertilized eggs while continuing to increase in the fertilized ones.

3. Activation, either spontaneous or artificial, brings about a rapid increase of the rate of uptake of the precursors used.

4. In the eggs of *Spisula* the rate of uptake of L-valine-1-C^{14} and of its incorporation into proteins is very low in the unfertilized eggs. A tremendous increase both in the rate of uptake and of incorporation into proteins takes place a few minutes after the fertilization-induced breakdown of the germinal vesicle.

5. The significance of these observations is discussed in relation to the problem of the release of the metabolic inhibition of the unfertilized egg upon fertilization, and in comparison with the results obtained in the sea urchin egg.

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